# Direct Measurement of Proinsulin in Human Plasma by the Use of an Insulin-Degrading Enzyme

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ABSTRACT A method has been described for the direct measurement of proinsulin in human plasma. The method makes use of an insulin-degrading enzyme designated "insulin-specific protease (ISP)", which is obtained from rat skeletal muscle. Under the conditions used, this enzyme rapidly degrades insulin and insulin-like polypeptides to nonimmunoassayable components, whereas proinsulin and proinsulin cleaved at position $B_{64,65}$ are not appreciably affected. The incubation of plasma with ISP results in the disappearance of insulin, but not proinsulin, as demonstrated by column chromatography. Immunoassay of the plasma, therefore, before and after incubation, determines the values for the total immunoreactive substance (TIR) and for immunoreactive proinsulin (IRP), respectively. The values obtained for proinsulin levels are reproducible and compare closely with the more complicated column fractionation methods.

Proinsulin responses were studied in four normal subjects and one patient with an insulinoma after a glucose load. Fasting proinsulin levels varied widely in the normal subjects, and the levels rose more slowly than TIR levels after glucose. IRP levels in the patient with an insulinoma were very high and fell to normal after removal of the tumor.

The ISP method, therefore, appears to be suitable for the direct, accurate, and rapid determination of proinsulin and proinsulin-like materials in human plasma.

## INTRODUCTION

The discovery of proinsulin, the single chain precursor of insulin (2), and the subsequent determination of its amino acid sequence (3) have led to much interest in the properties and distribution of this compound.

Although the presence of proinsulin and/or "big insulin" has been demonstrated in human plasma (4–7), its assay has been laborious and, in general, has required chromatographic separation of insulin and proinsulin followed by radioimmunoassay of each individual fraction. The difficulties inherent in such a complicated procedure have handicapped studies of proinsulin levels in plasma and have contributed to a lack of understanding of the role of this material in plasma.

This communication describes a method for the determination of proinsulin and proinsulin-like substances in plasma using an enzyme which specifically degrades insulin to nonimmunoassayable components without appreciably affecting proinsulin. The use of this enzyme allows the determination of proinsulin by a single incubation step followed by immunoassay for insulin, thereby circumventing the problems encountered in other proinsulin methods.

## METHODS

*Animals.* Male Holtzman rats (Holtzman Co., Madison, Wis.) weighing 200–300 g were used in these studies. The rats were fed Purina laboratory chow (Ralston Purina Co., St. Louis, Mo.) and had access to food and water up to the time of sacrifice.

*Chemicals.* Bovine plasma albumin (Fraction V, lot E30308) was obtained from Armour Pharmaceutical Co., Chicago, Ill. Human insulin, purified pork insulin, proinsulin, and C-peptides[1] were obtained from Eli Lilly and Co., Indianapolis, Ind. Polypeptides, related to pork insulin or proinsulin, were the gifts of Dr. R. Chance of the Eli Lilly and Co. Beef proinsulin was the gift of Dr. Donald Steiner of the University of Chicago. Insulin-$^{125}$I was purchased from Cambridge Nuclear Corp, Cambridge, Mass., and rabbit antiserum globulin to guinea pig serum was pur-

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[1] For definition of terms and source of compounds, see Reference 8.

chased from Cappell Laboratories, Downingtown, Pa. All other chemicals were obtained from commercial sources.

*Plasma samples.* Plasma samples were obtained from normal volunteers after an overnight fast by collecting blood in heparinized tubes, and immediately separating the plasma and pooling samples from five to seven subjects. The pooled plasma was separated into 2-ml portions and frozen. This plasma was designated "fasting plasma." 45–60 min after a 100 g carbohydrate meal, blood was again collected and the plasma pooled as above. These samples were designated "fed plasma." Glucose tolerance tests were performed as described before (9) by giving 100 g glucose orally and collecting blood in heparinized tubes at various times for determination of glucose, insulin, and proinsulin.

*Immunoassay of insulin, proinsulin, and related polypeptides.* The measurement of insulin and proinsulin was performed by the double antibody radioimmunoassay using a modification of the method of Morgan and Lazarow (10), as follows: Porcine insulin antiserum was produced in guinea pigs and used in the assay in a final dilution of 1:300,000. The incubation mixture consisted of 100 μl of plasma or standard, 50 μl of insulin-$^{125}$I (0.4 μCi/ml), 50 μl of 1:30,000 guinea pig antiserum, and 0.1 M borate buffer, pH 8.5, to a total volume of 0.5 ml. After incubation at 4°C for 24 hr, 50 μl of rabbit anti-guinea pig gamma globulin and 50 μl of a 1:25 dilution of normal guinea pig serum were added, and an additional 24 hr incubation was performed. The mixture was centrifuged, the antibody-bound insulin-$^{125}$I was washed, and radioactivity of the sample was determined in a Packard Autogamma spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill.). As shown in Fig. 1, the antibody used in this laboratory has similar affinities for pork insulin and related insulin polypeptides. The human insulin has reactivity similar to that of pork insulin (8). Although this antibody has less affinity for pork proinsulin and cleaved proinsulin than for insulin, the antibody still exhibits strong affinity for proinsulin. C-peptide has no reactivity on this immunoassay (8).

*Assay of protease activity.* Insulin-specific protease (ISP)[2] from rat skeletal muscle was prepared and purified by the method of Brush (11). Studies of enzyme activity and specificity were carried out in 0.1 M borate buffer, pH 7.5, containing 25 μl enzyme (8 μg protein), bovine plasma (3 mg/ml), and insulin, proinsulin, or other substrates ($10^{-8}$ to $10^{-11}$ M). Enzyme activity was also studied in the presence of human plasma by adding 100 μl of plasma with a low concentration of endogenous immunoreactive material to the incubation mixture and then measuring the disappearance of added insulin or proinsulin. In every case, the total volume of the incubation mixture was maintained at 225 μl. The reaction mixture was incubated at 37°C in a Dubnoff metabolic incubator and the reaction stopped by addition of 25 μl of 0.01 M N-ethylmaleimide (NEM). This mixture was assayed directly for immunoreactive insulin, as described above.

*Assay of proinsulin in human plasma.* Since pork proinsulin and split proinsulin have shown appreciable amounts of immunoreactivity by our insulin immunoassay using pork-insulin antiserum (Fig. 1), the total immunoreactivity by this procedure is composed of immunoreactive insulin plus immunoreactive proinsulin according to the following designations: TIR = IRI and IRP; TIR, total immunoreactive substance(s); IRI, immunoreactive insulin; IRP, immunoreactive proinsulin (or proinsulin-like compounds).

[2] *Abbreviations used in this paper:* IRI, immunoreactive insulin; IRP, immunoreactive proinsulin; ISP, insulin-specific protease; NEM, *N*-ethylmaleimide; TIR, total immunoreactive substance.

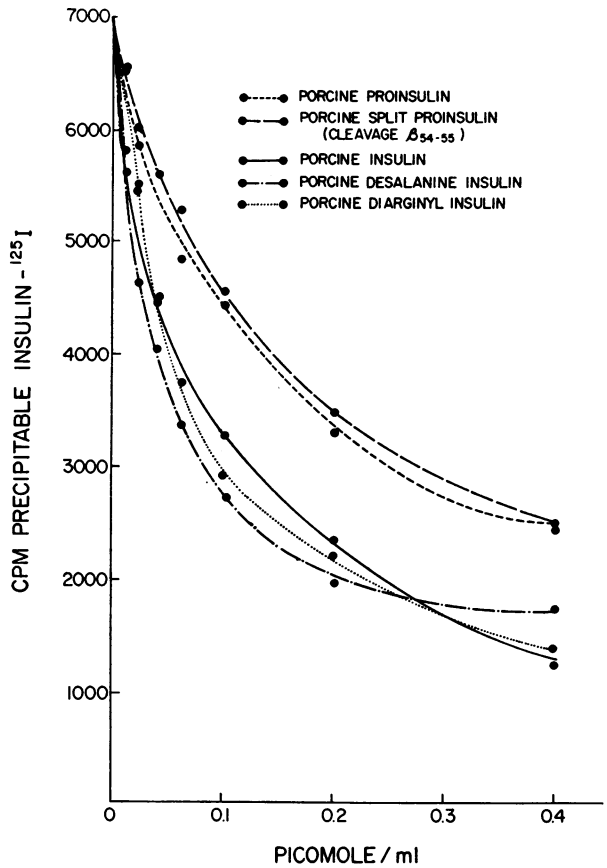


FIGURE 1 Immunoprecipitability of various preparations of insulin and proinsulin by the insulin double antibody immunoassay. The final dilution of insulin antiserum for 50% immunoprecipitability of labeled insulin is 1:300,000.

When proinsulin is determined by the ISP method, the following incubation mixture is used: 100 μl of plasma sample, 25 μl of enzyme (8 μg protein), borate buffer, 0.1 M, pH 7.5, in a total volume of 225 μl. At the end of 15 min of incubation, the reaction is stopped by addition of 25 μl of 0.01 M NEM. The mixture is then immunoassayed for insulin and proinsulin, as described above. When proinsulin is determined by column fractionation, the plasma is first extracted by a modification (12) of the method of Davoren (13) and then applied to a column of Bio-Gel P-30 (BioRad Laboratories, Richmond, Calif.).

## RESULTS

*Activity and specificity of insulin-specific protease.* A detailed study of the biochemical properties of the enzyme has been presented elsewhere by Brush (11). Studies presented in Figs. 2–4 demonstrate the kinetics of human, beef, and pork insulin degradation by ISP. Figs. 3 and 4 show that beef and pork proinsulin are relatively unaffected by ISP. Fig. 4 also depicts the

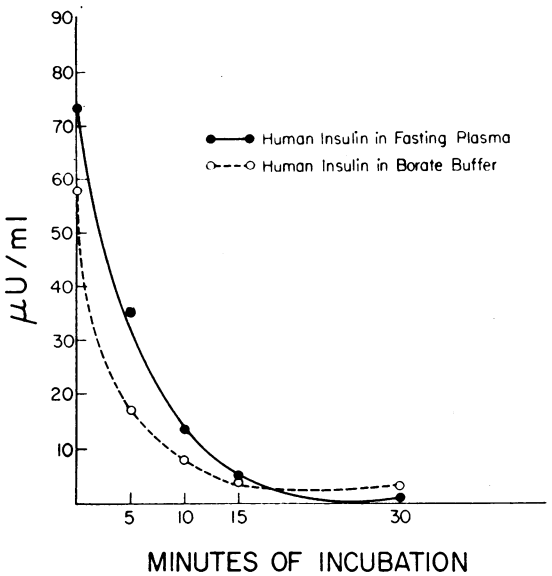


FIGURE 2 The disappearance of immunoreactive human insulin during incubation with ISP. Comparison of rate in plasma *versus* borate buffer. For procedure, see text.

kinetics of the destruction of compounds structurally related to insulin and/or cleaved proinsulin (14).

Desalanine insulin and diarginine insulin are degraded at a rate similar to insulin, while proinsulin with a cleavage at $B_{54,55}$ and proinsulin itself are degraded very slightly. Other proinsulin-like materials such as desdipeptide and desnonapeptide proinsulin do not appear to be degraded appreciably by ISP (15). This becomes important since the possibility exists that insulin-like materials in addition to insulin and proinsulin might be present in the circulation and might therefore interfere with the determination of proinsulin by the ISP method. If such materials do indeed exist in the circulation, the results obtained by the ISP method would include proinsulin-like material as well as proinsulin itself.

Fig. 5 shows that the proportion of insulin destroyed over a wider range of insulin concentration remains constant and that proinsulin is relatively unaffected. Table I demonstrates the destruction of variable concentrations of insulin added together with different concentrations of proinsulin. In each instance, the immunoreactivity remaining after incubation corresponds very well with the amount of proinsulin added. These studies confirm that ISP will destroy insulin in the concentrations commonly found in plasma and that the rate of degradation is unaffected by proinsulin in concentrations which might be found in plasma.

The possibility that other peptide hormones might interfere with the degradation of insulin was also considered. ACTH and growth hormone in a concentration of $3 \times 10^{-9}$ M and glucagon in a concentration of $3 \times 10^{-10}$ M failed to affect the rate of degradation. C-peptide similarly had no effect on the reaction.

Pooled normal plasma obtained as described under Methods, was incubated with ISP on several different days. Each incubation was performed on a separate portion from the same pool. As seen in Table II, a reproducible decrease in immunoreactivity resulted after incubation. This occurred with both high (fed) and low (fasted) concentrations of insulin. In addition, a single vial of pooled "fed" plasma was frozen and thawed five times. Portions were removed after each thawing and assayed for TIR and IRP. No significant difference was seen among the portions.

Although the standard incubation system contains 100 μl of plasma, smaller volumes can be used since proportional results are obtained with as little as 25 μl of

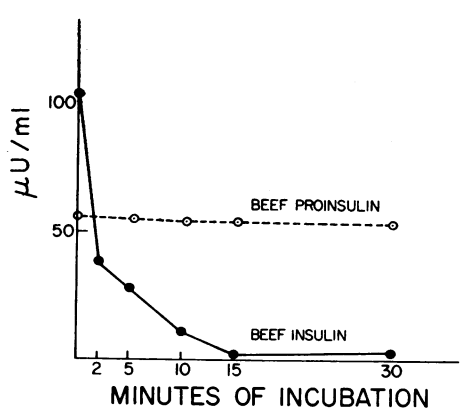


FIGURE 3 Degradation of beef proinsulin or beef insulin with ISP. Insulin or proinsulin has been incubated separately with ISP.

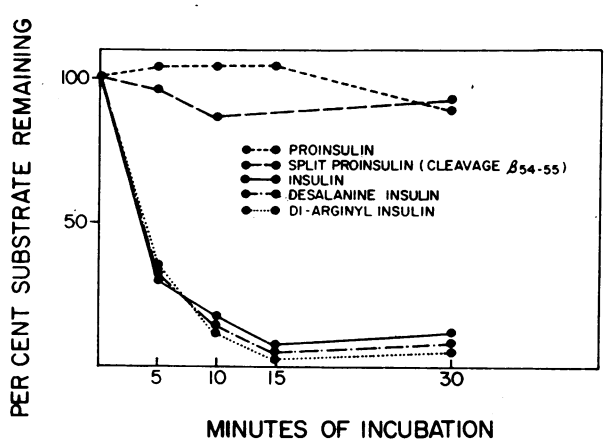


FIGURE 4 Comparison of the degradation of porcine proinsulin and a proinsulin derivative with porcine insulin and insulin-like materials. Each substrate has been incubated separately.

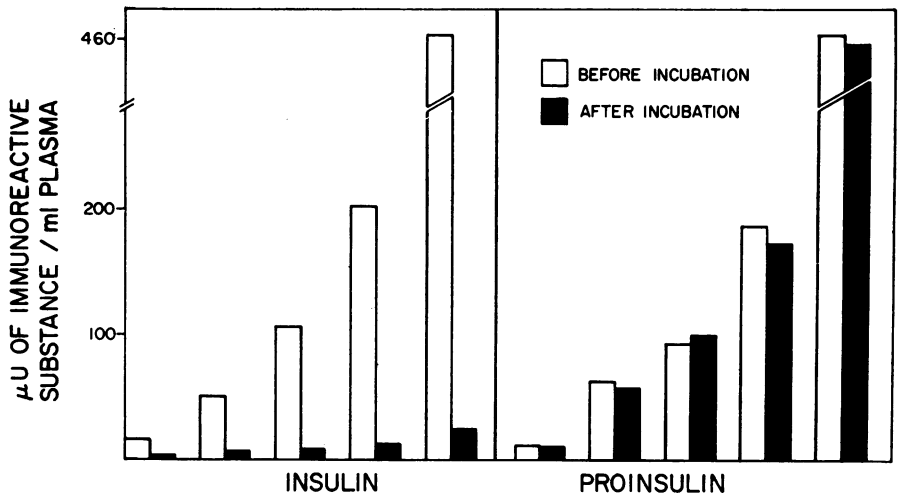


FIGURE 5 Demonstration of degradation of varying amounts of insulin by ISP and demonstration of lack of degradation of varying amounts of proinsulin.

plasma. This holds true for both "fed" and "fasted" plasma samples.

Table III shows that incubation of plasma without added ISP did not produce any change in immunoreactivity and that the insulin and proinsulin added to plasma before incubation could be recovered completely. This experiment demonstrates that plasma, in contrast to liver, kidney, diaphragm, and fat (16) has no insulin-degrading ability.

To prove that the decrease in immunoreactivity in plasma after treatment with ISP is due to destruction of the insulin, plasma was extracted with acid ethanol before and after incubation with the enzyme and then chromatographed on an 0.9 × 54 cm column of Bio-Gel P-30 which had been equilibrated with 3 M acetic acid. The protein was eluted with 3 M acetic acid and collected in 1-ml fractions. Immunoreactivity of the fractions was determined after removal of the acetic acid by drying. The values obtained for TIR and IRP by the ISP method were 38 and 15 μU/ml, respectively. The results obtained by chromatography can be seen in Fig. 6. The untreated plasma shows two peaks with a total immunoreactivity of 40 μU/ml. The proinsulin peak contains approximately 12 μU. After treatment with ISP, the peak corresponding to proinsulin contains 12 μU, while the insulin peak has disappeared.

In addition to the proinsulin peak, however, a discrete peak of material can be recovered in a late elution frac-

TABLE I

*Degradation of Insulin by ISP in Fasting Plasma in Presence of Varying Concentrations of Proinsulin**

| Insulin added | Proinsulin added | TIR | IRP |
|---|---|---|---|
| Control (plasma alone) | Control (plasma alone) | 10 | 2 |
| 5 | 5 | 19 | 7 |
| 50 | 5 | 64 | 4 |
| 200 | 5 | >200 | 5 |
| 5 | 50 | 68 | 53 |
| 50 | 50 | 110 | 48 |
| 200 | 50 | >200 | 55 |
| 5 | 100 | 117 | 114 |
| 50 | 100 | 156 | 109 |
| 200 | 100 | >200 | 102 |

* All values are expressed in microunits per milliliter.

TABLE II

*Replicate Analyses of Proinsulin and Insulin in Fasted and Fed Plasma*

| Sample number | Fasted plasma | | Fed plasma | |
|---|---|---|---|---|
| | TIR* | IRP‡ | TIR* | IRP‡ |
| | | μU/ml | | |
| 1 | — | — | 82 | 25 |
| 2 | — | — | 76 | 20 |
| 3 | 16 | 11 | 80 | 24 |
| 4 | 20 | 14 | 77 | 28 |
| 5 | 17 | 12 | 88 | 27 |
| 6 | 16 | 10 | 88 | 27 |
| Mean | 17.25 | 11.75 | 81.8 | 25.2 |
| SD | 1.9 | 1.7 | 5.2 | 2.9 |
| SEM | 0.9 | 0.9 | 2.1 | 1.2 |

* Total immunoreactivity of the plasma before incubation with ISP.

‡ Immunoreactivity remaining in the plasma after incubation with ISP.

TABLE III

*Recovery of Insulin and Proinsulin in Plamsa in the Absence of ISP before ($T_0$) and after 15 min ($T_{15}$) of Incubation*

| | $T_0$ | $T_{15}$ | Recovery |
|---|---|---|---|
| | $\mu U/ml$ | | % |
| Plasma alone | 14 | 14 | 100 |
| Plasma + insulin (*20 $\mu U/ml$*) | 34 | 38 | 111 |
| Plasma + insulin (*36 $\mu U/ml$*) | 50 | 50 | 100 |

tion, well after the fractions corresponding to insulin. This material appears to be of small molecular size and interferes with the binding of insulin-$^{125}$I and guinea pig anti-insulin antiserum in a manner similar to that of NEM (see below).

*Comparison of the method.* To compare the values for proinsulin measured by the present method with values measured by other methods (12, 17), plasma samples were obtained from the laboratories of Doctors Roth and Rubenstein. The insulin and proinsulin values, which were determined by column chromatography and subsequent immunoassay, were compared with the values obtained by our procedure. The results which are tabulated in Table IV demonstrate a relatively close agreement between the column methods and the present procedure. The variation in absolute values of TIR and IRP are not unexpected, since the use of different antisera by the three laboratories could result in different values for insulin and proinsulin.

*Factors influencing proinsulin assay by the ISP method.* Although the biochemical properties of ISP have been described elsewhere (11), the development

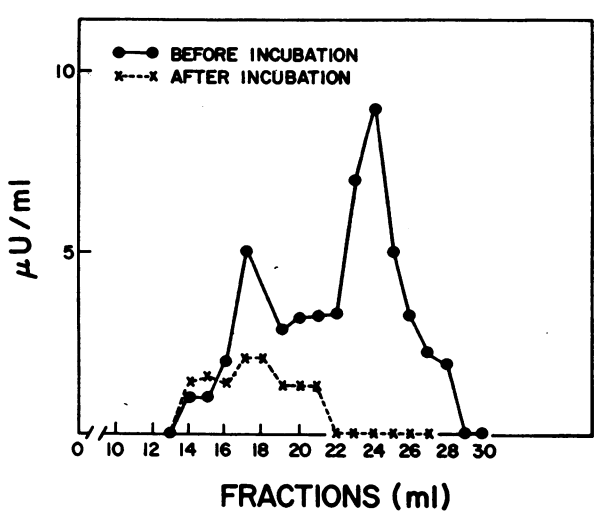


FIGURE 6 Fractions obtained from Bio-Gel P-30 column of extracted plasma before and after incubation with ISP. See text for detailed explanation.

TABLE IV

*Comparison of Proinsulin Values Determined by Different Methods*

| Sample | Fraction | Other methods | ISP method |
|---|---|---|---|
| A | TIR, $\mu U/ml$ | 40* | 65 |
| | IRP, $\mu U/ml$ | 17* | 28 |
| | IRP, % | 43* | 44 |
| B | TIR, $\mu U/ml$ | 60‡ | 74 |
| | IRP, $\mu U/ml$ | 27‡ | 44 |
| | IRP, % | 45‡ | 59 |
| C | TIR, $\mu U/ml$ | 113‡ | 132 |
| | IRP, $\mu U/ml$ | 41‡ | 57 |
| | IRP, % | 36‡ | 46 |
| D | TIR, $\mu U/ml$ | 70‡ | 61 |
| | IRP, $\mu U/ml$ | 8‡ | 8 |
| | IRP, % | 12‡ | 13 |

* For details of the method see Reference 12.
‡ For details of the method see Reference 17.

of a new incubation system, using this enzyme, required the study of factors influencing ISP actvity. In addition, the behavior of this enzyme was investigated in regard to (*a*) interference of ISP with the double antibody immunoassay, (*b*) the most effective method to inactivate the enzyme at the end of incubation so that labeled insulin used in the immunoassay will not be degraded, (*c*) optimum buffer and pH effect, and (*d*) the use of plasma *versus* serum for assay of proinsulin.

The following are the results of these studies: (*a*) ISP protein in its present state of purity (purified 5- to 20-fold) in concentrations of up to 50 $\mu$g of protein per 250 $\mu$l of incubation mixture will not interfere with the radioimmunoassay of insulin; (*b*) It was found after attempts were made to inactivate the enzyme by heat, by repeated freezing and thawing, by addition of silver nitrate, and by addition of NEM that the latter was the most effective method to inactivate ISP. The concentration of NEM, however, was critical, since NEM concentrations, an order of magnitude greater

TABLE V

*Effect of Varying Concentrations of N-ethylmaleimide (NEM) on per cent Immunoprecipitability of Insulin-$^{125}$I*

| Insulin standard | Control | 0.001 M NEM | 0.01 M NEM | 0.02 M NEM |
|---|---|---|---|---|
| $\mu U$ | | % | | |
| 0 | 67 | 67 | 30 | 26 |
| 20 | 46 | 46 | 25 | 20 |
| 40 | 39 | 39 | 21 | 16 |

TABLE VI

*Total Immunoreactive Substance (TIR) and Immunoreactive Proinsulin (IRP) During 100 g Oral Glucose Tolerance Tests in Four Normal Subjects*

| | Time, min | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 180 |
| J. M., 22-yr old female | | | | | | |
| Glucose, *mg/100 ml* | 95 | 145 | 115 | — | 112 | 104 |
| TIR, *μU/ml* | 12 | 76 | 82 | — | 41 | 22 |
| IRP, *μU/ml* | 9 | 10 | 12 | — | 12 | 11 |
| W. D., 28-yr old male | | | | | | |
| Glucose, *mg/100 ml* | 97 | 162 | 135 | 152 | 120 | 70 |
| TIR, *μU/ml* | 15 | 74 | 98 | 49 | 56 | 9 |
| IRP, *μU/ml* | 4 | 7 | 13 | 14 | 15 | 8 |
| H. H., 25-yr old male | | | | | | |
| Glucose, *mg/100 ml* | 96 | 139 | 129 | 105 | 106 | 75 |
| TIR, *μU/ml* | 6 | 43 | 54 | 50 | 45 | 11 |
| IRP, *μU/ml* | 3 | 13 | 14 | 12 | 15 | 4 |
| J. C., 22-yr old male | | | | | | |
| Glucose, *mg/100 ml* | 85 | 100 | 133 | 111 | 121 | 72 |
| TIR, *μU/ml* | 12 | 30 | 54 | 45 | 60 | 18 |
| IRP, *μU/ml* | 4 | 11 | 8 | 13 | 12 | 6 |

than 0.001 M interfered with the IRI assay. These results are illustrated in Table V, where the effects of 0, 0.001, 0.01, and 0.02 M NEM on the IRI assay are presented. In contrast to 0.001 M NEM, which had no effect on the standard curve, higher concentrations of NEM decreased the amount of immunoprecipitability (Table V). If larger amounts of NEM are used, a separate standard curve must be run, and values must be calculated from this; (*c*) Assays with ISP have been performed using Tris, phosphate, and borate buffers. Enzyme activity is similar in all systems. Since our immunoassay is performed in borate buffer, this buffer was chosen for the standard incubation system. Although ISP has a narrow range of pH optimum (7.4–7.6), the decrease in activity is less abrupt on the alkaline side. Because of this, and because the immunoassay is run at pH 8.2, we have chosen to run our assay at a pH more alkaline than the optimum. This pH is obtained by mixing borate buffer, 0.1 M, pH 7.5, with plasma. The incubation mixture can then be used directly for the double antibody immunoassay; (*d*) identical values for TIR and IRP were obtained from plasma and serum of the same whole blood sample. Addition of heparin to the serum did not affect the results.

*Physiologic studies.* Oral glucose tolerance tests were performed on four normal subjects (Table VI) and on one patient with a surgically and microscopically proven insulinoma (Fig. 7). It should be noted from Table VI that fasting proinsulin levels, as determined by the ISP method, vary from 26 to 75% of the TIR, and that proinsulin-like material may comprise considerably more than 50% of the fasting total immunoreactive substance. This table also demonstrates that the peak level of proinsulin occurs later than the peak level of insulin, thereby resulting in a higher percentage of proinsulin in the later specimens. Fig. 7 shows that a large (approximately 50%) proportion of the TIR in the plasma of a patient with an insulin-secreting tumor consisted of proinsulin. It can be seen, however, that the majority of the material released in response to a glucose load consisted of insulin-like material. Fig. 8 shows plasma TIR and IRP levels during and after surgery. Manipulation of the tumor resulted in an increase primarily of insulin, indicating also that the easily releasable material in the tumor consisted of insulin. After surgery, the levels of TIR and IRP returned to normal, demonstrating complete removal of the tumor.

## DISCUSSION

The importance and significance of proinsulin in human plasma are not as yet completely understood. Not only is there no clear pattern of abnormal response in disease states, but also the normal ranges and responses have not been completely elucidated. The primary rea-

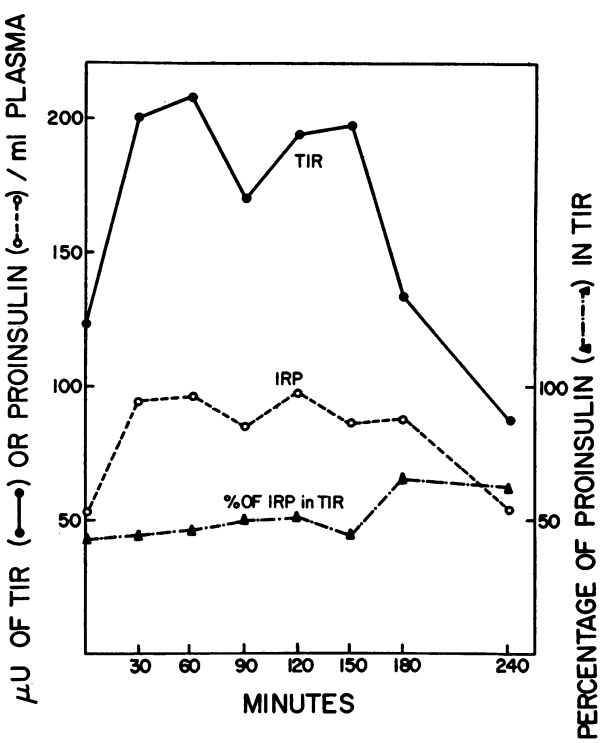


FIGURE 7 TIR and IRP values from a patient with a surgically proven insulinoma in response to a 100 g oral glucose load.

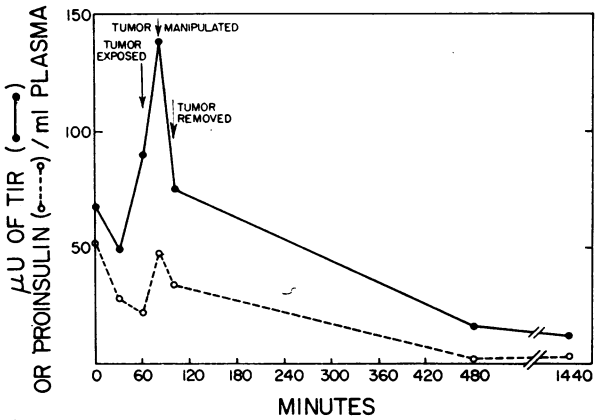


FIGURE 8 TIR and IRP levels in plasma before, during, and after removal of an insulin-secreting tumor.

son for this lack of knowledge has been the difficulty in measuring proinsulin levels. The gel-filtration method (4, 5) is long and laborious. The direct measurement of proinsulin has been handicapped by the difficulty of obtaining an antibody which is specific for proinsulin and which will not react with either insulin or C-peptide (14, 18). Further difficulty is encountered due to the species specificity of antibodies directed toward proinsulin (14, 18). Thus, the development of a specific antibody to human proinsulin must await the availability of purified human material. The discovery of an enzyme, therefore, which specifically degrades insulin but not proinsulin, provided an opportunity to develop a simple direct method for measuring proinsulin in plasma. Although numerous reports of insulin degradation in various tissues have been made (19–22), the effect of these enzymes on proinsulin had not been reported before the work of Brush, who showed the inability of ISP to degrade proinsulin appreciably (11). From the available biochemical studies, it appears that the insulin-specific protease reported here resembles the liver insulinase of Mirsky (19). Proinsulin studies, however, were not available in the earlier work of Mirsky. Studies reported now confirm the earlier report of Brush on the specificity of the enzyme and demonstrate its applicability to a proinsulin assay. The enzyme degrades insulin to nonimunoassayable components rapidly and completely. It does so at concentrations of insulin commonly found in human plasma and is not inhibited by proinsulin or other peptides. The values obtained for proinsulin by this method are reproducible and correspond quite well to values obtained by column fractionation, although there is a tendency to obtain slightly higher levels of proinsulin by the ISP method. This latter tendency is possibly explained by a greater contribution of the proinsulin intermediates to the ISP values as compared with the column method. Another possibility is that there is less manipulation of the plasma sample in the ISP method. A third consideration is that the antibody to insulin in our immunoassay may be less specific, and proinsulin, therefore, registers as a higher value when determined by our standard curve. In spite of this last possibility, it must be recognized that proinsulin has only one-fourth to one-third of the immunoreactivity of insulin with our pork insulin antiserum (Fig. 1) and that therefore the actual level of proinsulin is 3–4 times higher than the figures indicate. For determination of absolute levels of proinsulin, a proinsulin standard curve must be run.

Another factor to be considered in using ISP to determine proinsulin levels is the properties of the individual preparations of enzyme. In seven preparations of ISP, the range of insulin degradation has been 80–95%. Only those preparations which degrade more than 90% of the insulin substrate should be used for the assay.

The physiological studies described here in four normal subjects suggest that the level of proinsulin in normal subjects may not be as uniform as the level of insulin. Proinsulin values ranged from 26 to 75%. If one multiplies the proinsulin values which are obtained from insulin standard curves by 3 or 4, one cannot help but conclude that, in a certain number of "normal" subjects, the proinsulin may be a predominant immunoreactive substance in fasting plasma. The total explanation for this finding is not clear, but the slower turnover of proinsulin than insulin (23) may be one explanation. It would be interesting to follow subjects with normal glucose tolerance tests and with apparently normal TIR responses but with increased proinsulin/insulin ratios. It is possible that these subjects might be more prone to develop diabetes in later years. Obviously, a prospective study with prolonged follow-up will be necessary to prove or disprove such an hypothesis.

The method described here appears to be suitable for the direct measurement of proinsulin or its related polypeptides in human plasma. In addition, the lack of species specificity by this enzyme for its substrate makes the method applicable to other species and other tissue preparations. This method has been used recently to study proinsulin in the plasma of dogs (24).

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